STAIN TECHNOLOGY

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HISTORY OF THE COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS.[1]

H. J. CONN,

Agr. Exp. Station, Geneva, N. Y.

The present occasion seems a very good one upon which to review briefly the history of the investigations concerning stains with which this Commission has been identified.

In 1920 the secretary of the Society of American Bacteriologists asked its Committee on Bacteriological Technic, whose chairman was the present chairman of this Commission, to see if steps could be taken to procure a supply of bacteriological stains as satisfactory as the pre-war German stains had been. At that time the pre-war stains were still on the market, but the supply was rapidly dwindling. No new German stains could be imported, and the American manufacturers in general were turning out an unsatisfactory line. The committee looked into the matter and organized a cooperative investigation of the stains most used by bacteriologists. In this investigation various samples of these stains then on the market were compared. This investigation was carried on early in 1921, and the results were prepared for publication the following fall.

By this time it had become evident that the problem was too broad in its scope to be handled well by the bacteriological society; and the interest of the National Research Council was secured to organize the project on broader lines. Accordingly, on November 5th, 1921, a conference was called by the Chairman of the Division of Biology and Agriculture of the National Research Council, and was held at the Chemists Club, New York City. At this meeting there were present members of the division of Biology and Agriculture and of the Chemical Foundation, as well as others interested in the project. The Chemical Foundation offered to finance the undertaking, and a committee to effect the organization was appointed, with the chairman of the bacteriological society committee as its chairman. This new committee reported to the Research Council a

[1]Report of Chairman, at meeting of executive committee, at New York City, Nov. 20, 1925.

month later and recommended the appointment of a standing committee on Standardization of Biological Stains; which committee was then appointed under the Division of Biology and Agriculture. Later this same committee, with only minor changes, became the executive committee of the present Commission. Thanks to the assistance of the Chemical Foundation and to the interest aroused by the National Research Council, this committee was able to make considerable progress during the following year, and along much broader lines than if its work had remained an activity of the Society of American Bacteriologists.

By the fall of 1922 it had become evident that the committee on stains would find it difficult to proceed in its activities without committing the National Research Council in matters of a commercial nature. It seemed, therefore, wise to separate this work from the research Council; and the present Commission was organized. This Commission was composed of the fifty or more collaborators who had already been assisting in the committee's investigations, with an executive committee essentially the same in personnel as the committee appointed a year before by the Research Council. Formally the Commission is therefore a separate organization; but its relations with the Research Council are very close, and the interest of the latter body in the work is still as great as at the start.

Under this new arrangement all the advantages of the old organization were retained with none of its disadvantages. The Chemical Foundation still continued to finance the work, the Commission remained affiliated with the Research Council and the Treasury of the latter body continued to act as treasury for the Commission. At the same time the Commission is free to proceed in all matters without referring them to the Research Council and to publish its conclusions without obtaining the official endorsement of the Council.

Thus reorganized, the work proceeded in a very encouraging manner. The plan was soon adopted of certifying batches of stains submitted to the Commission for examination and found to be satisfactory. The batches of stains thus approved can be sold under a label bearing the certification of the Commission. By the beginning of the present year, certification had been issued on a considerable number of the stains, including all those for which there was the greatest demand. The chief obstacle up to this time had been that too long a period was required to test the stains submitted for certification. All the testing had to be done by volunteer work at laboratories widely distributed over the country, and it was natural that results should be obtained slowly.

These delays fortunately, have now been largely eliminated, thanks to the generosity of the Chemical Foundation. The appropriation which they have this year begun to give the Commission is enough larger than that of preceding years to bring about three innovations: the employment of an histologist to assist the chairman in his own laboratory in testing the stains submitted; the payment on a fair basis for work done in other laboratories; and the further appointment of a technician to carry on the routine parts of the chairman's duties to the institution at which he is located, and thus to release a large part of his time for the investigations on stains.

Results, therefore, are coming in more promptly than ever before. There are now very few biological stains that have not been put on the certification basis, except those which are very seldom used by biologists. All the important stain manufacturers are now cooperating with the Commission and are sending in stains for testing and approval. A brief hand-book on the nature and uses of biological stains has just been published by the Commission;[2] and out of an edition of 1200 copies, 500 have been sold before publication. Hence this book will become, not a liability, but an asset to the finances of the Commission. By a happy coincident the first distribution of copies of the book, and hence the actual date of publication, occurred on November 5th, four years to a day after the organization of the work under the National Research Council.

The Commission can now safely claim, therefore, without seeming boastful, that it has already made a distinct contribution to Science, and that further contributions are likely to come from the work in the future. It is also safe to say that without the financial assistance of the Chemical Foundation and the active interest of its president only a very small part of what has actually been accomplished would have been brought about. It is, accordingly, the opinion of the chairman that on this occasion the gratitude of the Commission should be officially expressed, not only to the Chemical Foundation, but also to its president, Mr. Garvan, personally.

Following this report, the following resolution was made and adopted by the executive committee:

Resolved, That the thanks of the Executive Committee of the Commission on Standardization of Biological Stains be extended to the Chemical Foundation and to its president, Mr. F. P. Garvan, for their interest in the establishment of the Commission and their assistance in the prosecution of its work.

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[2]H. J. Conn, Biological Stains. Geneva, N. Y., 1925.

CONSTITUTION OF COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

(As Amended December, 1923)

PREAMBLE: In 1921 the National Research Council began an undertaking looking toward the standardization of biological stains and carried on the work connected therewith thru a committee appointed by the Division of Biology and Agriculture of the said National Research Council. The National Research Council and all others interested desire that the work undertaken by such Committee, at the instance of said National Research Council, shall continue. However, for divers reasons, said Committee must thereafter operate independent of the National Research Council and it becomes necessary, therefore, to form a new organization to carry on the work. In forming this new organization it is the aim and desire of all who have been associated with the said undertaking, to organize in such a way as to permit the continuance of the work with as little interruption as possible, and therefore the form of organization outlined in the articles following is submitted:

ARTICLE I. NAME: The name of this organization shall be the Commission on Standardization of Biological Stains.

ARTICLE II. OBJECT: This Commission is a non-commercial organization formed on the understanding that no member is to receive any private profit from the sale of any product of the character dealt with by the organization nor is to be in any way financially interested in the endorsement of any such product by the Commission.

The primary object of this organization will be that implied in its title, namely the standardization of biological stains. It shall also have the following objects which will all contribute toward the same end:

1. Carrying on investigations looking toward the perfecting of the supply of biological stains and toward the development of new uses for them.

2. Bringing about cooperation between manufacturers, dealers and users of stains so as to carry out the lines suggested by the results of these investigations.

3. Publishing scientific data relating to the nature and use of biological stains.

4. Preparing statements to familiarize the public with stains and

their uses and to explain to users of stains the most satisfactory sources of supply and best methods of using the different products.

5. Issuing statements of certification to manufacturers, which they may use in accordance with and subject to the rules and regulations from time to time adopted by the Executive Committee of the organization, on different batches of stain that have been tested by the Commission.

6. Carrying on investigations in related fields wherever they seem desirable toward bringing about any of the objects of the organization.

ARTICLE III. MEMBERSHIP: The members of this organization shall be those of the collaborators secured by the Committee on Standardization of Biological Stains (appointed in 1921 by the National Research Council) who endorse this Constitution. Other persons shall be eligible to membership who establish to the satisfaction of the Executive Committee that they are interested in the work of the organization and are ready, willing and able to assist in the work of the organization, if and at such times as assistance in his or her special field may be requested of such member by the Executive Committee. Any such person may be admitted to membership and upon his application for membership receiving the approval of the members of the Executive Committee. Continuance of membership shall be evidenced by the members signing and returning to the Chairman of the Executive Committee semi-annually a declaration in substantially the following form: "I desire to renew and continue my membership on the Commission on Standardization of Biological Stains and desire my name and address to be continued on the list of members." Blanks for this purpose shall be sent to all members of the organization semi-annually and shall be returnable within one (1) month. Any member failing to sign and return two (2) consecutive blanks shall be dropped from the membership roll.

SOCIETY REPRESENTATIVES: Any national scientific society so desiring may elect or appoint a representative to the Commission. This representative then becomes a member of the Commission with the additional duties of keeping his Society posted as to the work of the Commission and of representing to the Commission the viewpoint of his society in matters of interest to both organizations.

The election or appointment of said Society representatives shall be construed as giving the Commission the authority to undertake and to carry on the work for which the organization is created under the endorsement of the respective societies thus represented.

ARTICLE IV. EXECUTIVE COMMITTEE: The affairs of this organization shall be managed by an Executive Committee of five members. The original membership of said Executive Committee shall be the five members representing respectively the following societies:

Society of American Bacteriologists.
Botanical Society of America.
American Chemical Society.
American Association of Pathologists and Bacteriologists.
American Society of Zoologists.

These five members shall hold office until the election or appointment of their successors by their respective societies. When any of the above named organizations appoints a new representative to the Commission, the new representative does not, however, become automatically a member of the executive committee. The former representative of that Society shall continue to serve as a member of the executive committee until its next meeting. At said meeting the members present shall by majority vote elect a successor to the retiring member, the candidates for the vacancy being the society representatives then on the Commission who are not members of the executive committee.

The first Chairman of this Executive Committee, who shall also be Chairman of the Commission, will be the man appointed in 1921 by the National Research Council as Chairman of the Committee on Standardization of Biological Stains. His term of office will be two years, beginning the first day of May 1923. Upon the expiration of this term of office the position shall be filled as follows:

At the last meeting of the Executive Committee preceding the expiration of the term of office of any chairman of said Committee, a member or two members, of said Committee shall be nominated as a candidate or as candidates for Chairman for the following two years. The nomination or nominations shall be submitted to the members of the Commission, and an election conducted as follows:—Within thirty days following the meeting of the Executive Committee at which said nomination or nominations are made, and at least two weeks prior to the first day of May, ballots shall be mailed to all enrolled members at their last known addresses on which shall be printed the name of the candidate or candidates nominated by the Executive Committee and a blank space for writing in the name of any other member of said Committee for which a vote is to be cast instead of for a candidate nominated by said Committee. The polls shall be open from the day of mailing said ballots until 10 o'clock P. M. on the day of the election as specified in the ballots. The

ballots shall be opened by a teller selected by the Executive Committee, who will declare the person receiving the largest number of votes cast to be elected Chairman of the Executive Committee. The member thus elected will take office on the first day of May following election.

The term of office shall be two years, the first election occurring in April 1925 on a date fixed by the Executive Committee at the time the nomination is made and subsequent elections every second year thereafter on a date similarly fixed by the Executive Committee. In the event of a vacancy of the Chairman, the Executive Committee shall elect a Chairman to hold office until the next regular election.

The Executive Committee shall have the right at any time to select one of its members to fill any one of the following offices if such offices appear to them to be necessary for the affairs of the organization: Vice-Chairman; Secretary; Treasurer.

The duties of the Executive Committee shall be to pass on the ordinary affairs of the Commission and to approve reports and other matters for publication. The duties of the Chairman of the Executive Committee shall be to initiate matters of business, to submit reports to the other members of the Executive Committee for approval, to keep in touch with the entire membership of the Commission and with organizations and individuals with whom the Commission is co-operating and, subject to the authorization, direction and approval of the Executive Committee, to issue statements of certification to manufacturers. He shall take charge of the correspondence and financial matters of the Commission until such time as the Executive Committee decides to appoint a Secretary and Treasurer to take care of such matters. It shall also be his duty to see that reports are sent out to the entire membership of the Commission at least twice a year.

ARTICLE V. FINANCES: Money placed at the disposal of the Commission shall be deposited with some fiscal agent selected by the Executive Committee, but with the understanding that if at any time the Commission becomes incorporated and decides to handle its own money it may discontinue this policy and maintain its own treasury; whereupon said fiscal agent shall, upon such incorporation of this organization, turn over to the Treasurer of this organization whatever funds may then be in control of said fiscal agent deposited for the use of this organization. Funds for the support of its work may be obtained from special donations, and from fees charged to commercial concerns to whom services are rendered.

ARTICLE VI. AMENDMENTS. Amendments of this constitution may be adopted upon a majority vote of the entire membership, after having first met the approval of the Executive Committee. Voting upon amendments shall be by ballot and shall be conducted by mail. The proposed amendment with ballot shall be mailed to all enrolled members at their last known addresses not less than six weeks prior to the date upon which the vote is to be taken, which date shall be specified in the ballot, and the polls shall be open from the day of mailing of the proposed amendment and ballot until ten (10) o'clock P. M., of the day set for the taking of the vote. The ballots cast shall be counted by the teller selected by the Executive Committee and all ballots not returned shall be counted as voted in the affirmative. The teller shall render a report to the Executive Committee and declare the amendment either carried or not carried as the case may be, and a copy of the teller's report shall thereupon immediately be mailed to every enrolled member of the organization at his last known address.

PRESENT MEMBERS OF THE COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

Executive Committee

H. J. Conn, Agricultural Experiment Station, Geneva, New York
R. W. French, Walter Reed General Hospital, Washington, D. C.
S. I. Kornhauser, University of Louisville, Louisville, Ky.
F. B. Mallory, Boston City Hospital, Boston, Mass.
L. W. Sharp, Cornell University, Ithaca, New York

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STANDARDIZATION OF BIOLOGICAL STAINS AS A PROBLEM OF THE MEDICAL DEPARTMENT OF THE ARMY[1]

R. W. FRENCH[2]
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ABSTRACT.—**Since government procurement necessitates comprehensive specifications entirely free from elements of personal equation, the study of dyestuffs for biological stain and therapeutic uses was undertaken in the Medical Department, U. S. Army, early in 1924. Work has been carried on since that time and specifications for the purchase of hematoxylin and eosin Y have been submitted. A number of other dyes have been studied, but the results to date indicate a complex problem which will probably require extensive research for its consummation. Spectrophotometric methods of study are being developed specially in this study and that instrument is being found specially valuable in the study of dyestuffs.**

With the constantly growing demands for bacteriological and pathological science in connection with the practice of medicine, the Medical Department of the Army has an increasing need for reliable biological stains. As in other governmental agencies, procurement is centralized and, what is more important in appreciating some of the difficulties to be surmounted, purchase must be made from the lowest bidder, other things being equal.

This last situation has made it possible for the foisting onto the government of many undesirable products on account of the fact that procurement specifications were wholly lacking or markedly inadequate. It has been almost impossible to provide comprehensive specifications for the procurement of dyestuffs for biological stain use which would be devoid of the personal equation in the testing of samples. This latter feature is an essential, as manufacturers seeking business are hardly likely to concur as to who is really a qualified histologist or bacteriologist in making a given test under trial and test methods of specification.

Prior to the World War the need for specification requirements in this field was not so apparent, due to the fact that Dr. Grübler was practically the only source of dyes for biological stain purposes and

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[1]Contribution from the Laboratory Service, Walter Reed General Hospital, Army Medical Center, Washington, D. C.

[2]Author's Note: This work has been carried on under the direction of Major R. E. Scott, Medical Corps, U. S. A., and Major C. G. Sinclair, Medical Corps, U. S. A. Chiefs of the Laboratory Service, Walter Reed General Hospital.

almost automatically dyes were procured from that source, either directly or indirectly. However, since the World War, with the advent of numerous manufacturers and dealers, difficulty has appeared in obtaining reliable dyes from lowest bidders without adequate specification.

While the work of the Commission on the Standardization of Biological Stains has accomplished a great deal in improving the general quality of dyes available for biological stain purposes, its work could not be used under governmental requirements. Clauses such as the following, which occur in the Commission requirements, are not allowable in a government specification as admitting too personal an element: "The sample should prove satisfactory for histological use. No exact methods for determining this can be given but the sample must be submitted to one or two experts in histological technic in order to get their judgment."[3] Such a requirement leaves entirely too much of a loop-hole for certain unscrupulous bidders looking for government business. It is not practical for the government to demand a certified stain; but under the law, it may demand a certified stain or its equal. Now, in the lack of specifications, there is nothing to prevent a bidder stating that his product, though cheaper, is fully as good as the certified dye, but that it has not been certified because it was not submitted to the Commission for such certification. If specific requirements are met, and if the bid is the lowest, this dye will have to be purchased under existing regulations.

To meet this intolerable situation a study of dyes for biological staining purposes was undertaken in this laboratory in the Spring of 1924 and has continued to date. The earliest studies disclosed the fact that the problem was extremely complex and one which would easily require a great deal of research for any approximation of consummation. Several factors were noted which need standardization: first, the multitude of technics, specially with reference to histological methods; and secondly, the marked variations in dyestuffs as produced by the same and different manufacturers. Both factors have an inter-relationship which precludes a separate discussion.

Again, both of these primary considerations have several phases which require study. With reference to technic and application the following questions occur:

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[3]From the Commission requirements for a certified Safranin. See Conn, Biological Stains, Geneva, N. Y., 1925, p. 133.

What is the influence of fixation? What is the action of different fixing agents?
How does the dye in question act? What influences its action, specially with reference to hydrogen-ion concentration?
Exactly what dye is necessary or desirable?
In the case of dye combinations, are these combinations logical in the face of the chemistry of the dyes concerned?

When it is realized that many of our technics have been developed by workers entirely unacquainted with dye chemistry or the slightest chemical knowledge of the dyes involved, it is surprising that more of the technics are not of doubtful value.

With reference to the variations in dyestuffs from the same and different sources, it is but natural, in view of the complexity of dye chemistry, to expect great variability. It requires, however, a careful comparative study of the several products in the light of the above factors to determine which may be advisable. This is further complicated by the fact that many dyes, as produced, are not chemically pure, but admixed with other dyes, and at times study has shown that in reality these impurities are of greater import than the principle component. These variations make reliable means of examination and analysis necessary.

On the assumption that a fixed chemical composition should result in a fixed color, the spectrophotometer was adopted as a means of dye analysis thru the absorption spectrum of the dye solution. Practice has shown this assumption to be essentially true. However, spectrophotometry is new and many inaccuracies in the analysis of dyes have been noted, due to solvent, concentration, etc. As these fundamentals are determined, it is believed that this instrument will prove of inestimable value in such analyses, both qualitative and quantitative.

Specifications resulting from the study have been submitted on two dyes, Hematoxylin and Eosin Y.[4] Other dyes which have been studied are: Bismarck Brown Y, Brilliant Green, China Blue, Crystal Violet, Dimethylaminoazobenzene, Fluorescein, Basic Fuchsin, Gentian Violet, Giemsa Stain, Methylene Blue, Methyl Violets, Scarlet Red (Sudan IV), Sudan III, Thionin (Lauth's Violet), Wright's Stain, Neutral Acriflavine, Mercurochrome, 220-Soluble, Orange G, Methyl Green, Pyronin, Hematin, Erythrosin, Rose Bengal, Brilliant Cresyl Blue, Azure I, Azure II, Methylene Violet (Bernthsen), Azure-II-Eosin, Bromsulphalein, Acriviolet, Azure A, Azure B, Azure C and MacNeal's Tetrachrome Stain. Of these, Neutral Acriflavine, Acriviolet, Mercurochrome, 220-Soluble, and

[4]Scott, R. E., and French, R. W., Military Surgeon; November, 1924.

Gentian Violet have been studied with special reference to therapeutic use.

Some of the studies are nearing completion, while many await extensive chemical work. Methylene Blue was one of the first dyes to be considered, and the work has been carried on continuously since April 1924. At the present rate of progress it is hoped to have the study of this and allied dyes completed within the next year. It is undoubtedly one of the more complex dyes employed by the biologist and subject to some remarkable variations in the ordinary processes of manufacture. Some of its homologs appear to have special value[5] and it is believed that in reality the occurrence of these homologs as impurities gives Methylene Blue its value as a tissue and bacteriological stain.

Inter-twined with the problem of Methylene Blue is the study of numerous compound stains such as MacNeal Tetrachrome, Giemsa and Wright's stains. The former offers one advantage—it is a definite mixture of certain dyes, to wit: Methylene Blue, Eosin, Methylene Azure (MacNeal), and Methylene Violet (Bernthsen). Wright's, Leishman's, Giemsa stains etc., are found to be ordinarily polyglot mixtures of doubtful composition. In general it proves that the alkaline oxidation of Methylene Blue is unreliable and that resultant products will exhibit marked variations.

In the study of the application of Methylene Blue and allied stains it was early noted that hydrogen-ion concentration played an important role. The employment of suitable buffers, for instance, is found to enhance materially the results obtainable with the compound blood stains.[6] Buffer control also is an essential in certain other stains (Holmes and French in this JOURNAL, p. 25) and it is believed that as study permits, it will be found to be an essential to consistent and dependable results in general staining technic.

Gentian Violet and allied dyes are likewise a complex problem. The products on the market, both of American and foreign origin, are not duplicates of pre-war dyes, either as regards dye content (which was notably low before the World War), or with reference to chemical nature. This difference, while not of quite so much import when these dyes are considered as staining agents, is of special importance when therapeutic utility is considered. One sample of pre-war Gentian Violet, for instance, has been found to be three and four times as powerful bactericidally as others with which it has been compared. Present dyes also fail noticeably when employed for

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[5]Holmes, W. C., and French, R. W., Stain Technology, current issue, p. 17–26.
[6]Scott, R. E., and French, R. W., Military Surgeon, August 1924.

decortication in surgery. Several factors require consideration, first the fundamental difference as to base, para-rosaniline or rosaniline, and second the effect of varying degrees of methylation. Some work has been carried on along these indicated lines, but on account of limited chemical facilities it has not been possible to do more than barely indicate probabilities.

In general, the work is being conducted along what is believed to be constructive lines and with completion it is hoped that the dye manufacturers will have information as to what dyes are desirable and how they may be economically manufactured of requisite purity. On the other hand, it also is hoped that some information will be gleaned as to the manner of action of the dyes in accomplishing staining and therapeutic effects. Considering that dyes employed in science represent a very small porportion of the total dye business in dollars and cents, it is readily realized that the dye-makers are not in a position to assist materially in a study which may require a great deal of abstract research for its proper consummation. This fact also has a tendency to make progress slow, as few chemists or few laboratories are equipped to carry on an extensive study in dye chemistry outside of the factories.

Ultimately it seems that with standardization accomplished the following facts should be patent:

1. The biologist should know precisely what dye or mixture of dyes he requires for a given purpose.
2. If a mixture, he should know exactly what components are necessary and how much of each.
3. The relative action of so-called fixing agents and the dye should be known.
4. The need of the so-called mordants should be established.
5. The dye chemist should know definitely the dye desired by the biologist and a reasonable method of manufacturing with a requisite degree of purity in the case of each and every desirable dye.
6. With standardization accomplished there should be certain intelligible specifications available for each dye, specifications which the manufacturer can be reasonably expected to meet.
7. There also should be standard methods of use, so that staining results with a given dye, or technic, may always be thoroly comparable.
8. If possible, the nature of the action of the dye should be determined. In the light of our present knowledge of dyeing, or the stain effect of a dye, it may be little likely that this will be generally understood, tho it is probable that it will become evident in numerous instances.

The supplying of these facts no doubt may be considered an ideal of attainment, but one worthy of the hire, when it is realized that probably ninety per cent of the facts known through the application and use of the microscope in science are due solely to the stain effect of dyes. Dyes may be aptly considered one of the very foundation stones of microscopical science. The time has arrived when it would be well if more were known regarding their function and action, in order that logical scientific advancement may supercede the random processes of the past in the employment of these invaluable aids to the bacteriologist, the pathologist, and to all microscopists.

THE OXIDATION PRODUCTS OF METHYLENE BLUE

W. C. HOLMES, *Color Lab., Bureau of Chemistry,* and R. W. FRENCH, *Walter Reed General Hospital, Washington, D. C.*[1]

ABSTRACT.—**The mechanism of the oxidation of methylene blue varies with the conditions. The formation of trimethyl thionin (azure B) and of asymmetrical dimethyl thionolin (azure A) is followed under alkaline conditions by that of dimethyl thionin (methylene violet) and under acid conditions by that of monomethyl thionin (named by authors azure C).**

Simple and practical methods are given for the preparation of azure A and azure C. The latter product, which has not been obtained from methylene blue hitherto, has valuable staining properties as a nuclear and bacterial stain in tissue and may also be employed satisfactorily as a substitute for azure A in the MacNeal tetrachrome formula as a blood stain or substitute for the Giemsa stain.

Azure B has no particular merit in staining.

Azure C proves to be a very valuable stain. A procedure is given for its use with eosin Y and orange II as counterstains, by which it is possible to demonstrate bacteria in tissue and at the same time the cytological elements of the tissue.

Shortly after the introduction of methylene blue in microscopic practice it was noticed that aqueous solutions of the dye gradually undergo changes which give rise to polychrome staining effects. It was found, further, that these alterations were the result of oxidation and were greatly accelerated and accentuated by the presence of alkalies. In the subsequent bacteriological and pathological technic which has developed, the employment of alkalies in the application of the dye has been somewhat general.

With staining formulae of this type, the results obtained by different workers have varied greatly, and the individual worker has found it impossible to insure consistent operation. Because of these uncertainties, manufacturers of stains have attempted to supply the biologist with the essential polychrome agents in concentrated and convenient form to enable him to prepare solutions of a more definite composition. For this purpose methylene violet and methylene azure (azure I) have been marketed.

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[1]Contribution from the Color Laboratory of the Bureau of Chemistry, United States Department of Agriculture, jointly with the Laboratory Service, Walter Reed General Hospital, Army Medical Center, Washington, D. C.

Methylene violet (Bernthsen) is dimethyl thionolin, or methylene blue in which one dimethylamino group has been replaced by a hydroxyl group (to be distinguished from the textile azine product listed in the Colour Index under No. 842). It is a constituent of MacNeal's tetrachrome stain[2] but its general utility is less than that of certain azures. We have duplicated MacNeal's preparations of this dye but have not attempted any further investigation, as the market supply appears satisfactory.

Methylene azure is defined in the Colour Index as a trimethyl thionin (the azure B of the biologists) containing some asymmetrical dimethyl thionin (azure A). These constituents are related to methylene blue through the replacement of one and two methyl groups, respectively, by hydrogen atoms. In our experience commercial samples of the product have seldom conformed to the definition cited. They have proved extremely variable in composition and unsatisfactory in staining operation.

The present investigation was undertaken in the hope of extending our knowledge of the oxidation of methylene blue and of obtaining from it products which would have valuable staining characteristics and be reasonably homogeneous.

Of the earlier investigations in that field the most important were those of Bernthsen[3] and of Kehrmann[4] and his collaborators. All of the compounds to which we shall here refer have been prepared by synthetic methods, and considerable information is available in the literature on their chemical properties and absorption. Their absorption bands are well separated in spectral location (See Chart 1) and we have relied principally upon spectrophotometric examinations in determining both the character and the degree of homogeneity of our products. Supplementary evidence was obtained by extractions with immiscible solvents. The compounds of the thionin series are strongly basic; those of the thionolin series are only slightly basic; whereas thionol, which is related to thionin through the replacement of both amino groups by hydroxyl groups, is distinctly acid. The individual members in both the thionin and thionolin series vary appreciably in solubility.

The principal products of mild alkaline oxidations of methylene blue were found by Bernthsen and by Kehrmann to be azure B,

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[2]MacNeal, W. J., J. Infect. Diseases, **36**, 538, 1925.
[3]Bernthsen, A., Liebig's Ann. de. Chimie., **230**, 73. 1885.
Ber. d. Deut. Chem. Gessell., **39**, 1804. 1906[2]
[4]Kehrmann, F., Ber. d. Deut. Chem. Gessell., **39**, 1403. 1906.
Ber. d. Deut. Chem. Gessell., **46**, 2131. 1913.

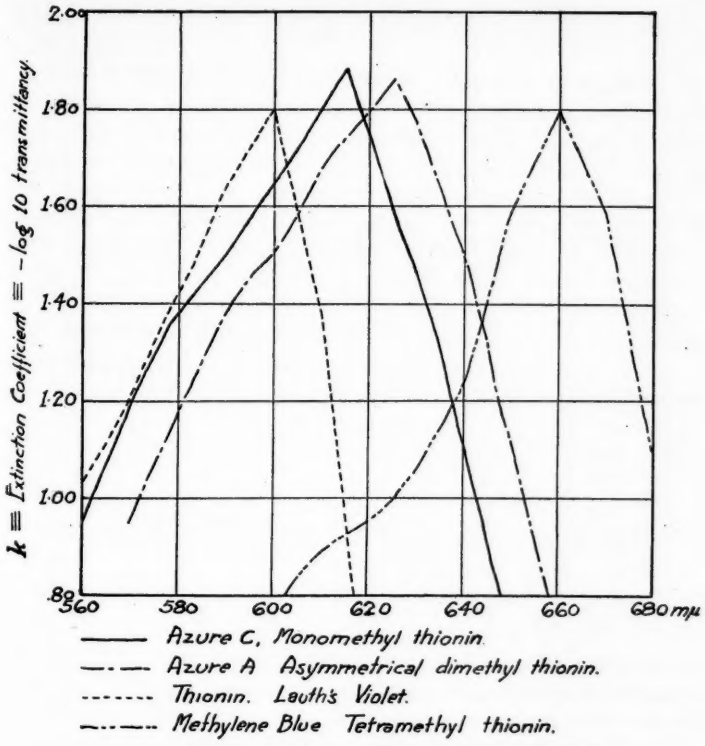


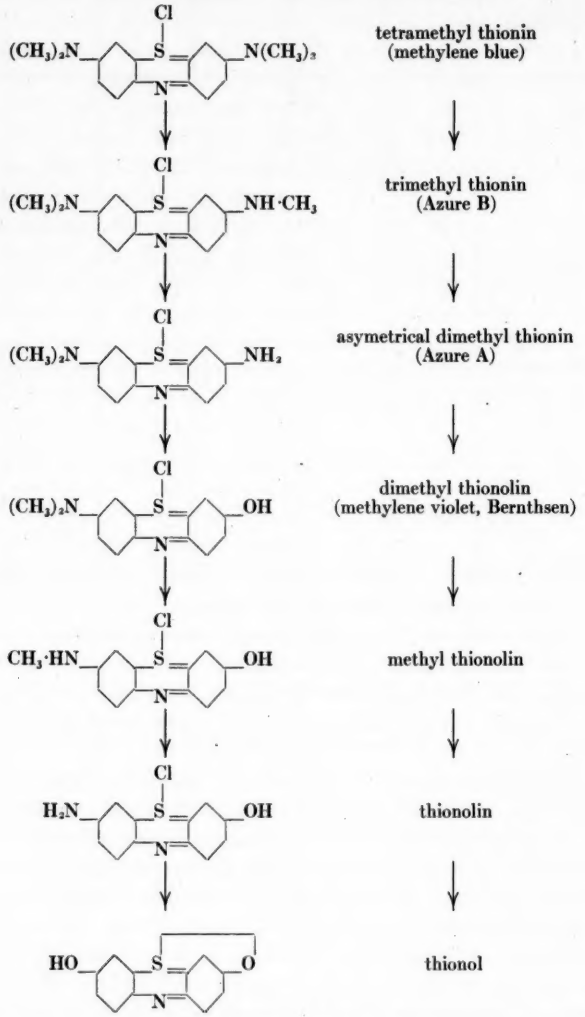
CHART 1. ABSORPTION CURVES OF AZURES A–C, OF THIONIN AND METHYLENE BLUE—[French]

azure A, and methylene violet. Under more drastic conditions methyl thionolin, thionolin, and thionol are formed. Our results with alkaline oxidation have confirmed these conclusions. (See Chart 2.) Still other products of unknown constitution may result, however.[5]

Alkaline oxidations have invariably resulted in decidedly heterogeneous mixtures containing hydrolysed products as well as azures, or lower homologs of methylene blue, and we have definitely rejected the employment of alkalies in the preparation of azures. The results obtained are too greatly affected by conditions which are not easily controlled, and the products appear to be peculiarly susceptible to further oxidative alteration of an unfavorable nature encountered in drying them, particularly when large quantities of material are being handled.

[5]Scott, R. E. and French, R. W., Military Surgeon, Sept. 1924.

CHART 2. ALKALINE OXIDATION OF METHYLENE BLUE



Very little information is available in the literature concerning the oxidation of methylene blue under acid conditions. By treatment with acid and bichromate, MacNeal obtained an azure A which was a comparatively homogeneous product of excellent staining characteristics, and an impure azure B, which gave no promise of practical

utility. His methods of preparation were not well adapted for large-scale operation.

In the following series of experiments 5 grams of methylene blue were dissolved in 200 c.cm. of water and the solution brought to a boil. (The dye employed was a zinc-free textile product of the National Aniline and Chemical Co., containing 72% actual dye.) To the boiling solution was added a hot solution containing 10 cc. of concentrated (36%) hydrochloric acid in 100 cc. of water, together with a quantity of potassium bichromate which varied in the different tests. The combined solutions were boiled (under a reflux condenser) for 45 minutes. The quantities of bichromate and the absorption maxima of the oxidized solutions at the conclusion of the boiling are recorded below:

Test No.	$K_2Cr_2O_4$ Grams	Absorption Maxima mμ
1	.5	658
2	1.0	650
3	1.5	645
4	2.0	635
5	2.5	625
6	3.5	618
7	5.0	615

The absorption maxima were determined by the spectrophotometric method. The decreasing values indicate the progressive oxidation and demethylation of the dye. The maximum of methylene blue is approximately 662 mμ; that of the completely demethylated dye (thionin, or Lauth's violet) is about 598 mμ; while that of MacNeal's azure A is about 623 mμ. (See Chart 1.)

The complete spectrophotometric data indicate that the solutions contained a mixture of dyes in each instance. In distinction to alkaline oxidation, however, the products were exclusively azures and without admixture with methylene violet. Apart from this the most interesting feature of the results is the evidence they afford that azure formation continues in acid solutions beyond the point at which two methyl groups have undergone replacement.

The most homogeneous product was obtained in test No. 5. The oxidized solution obtained in that test was practically identical in character with the corresponding solution obtained by following MacNeal's specifications for azure A. When these solutions were salted out, appreciable quantities of azure B remained in solution and the resulting azure A was of fair purity. It contained appreciable amounts of both the higher and lower homolog, however. In our

opinion the troublesome and expensive after-treatment with alcohol which MacNeal specifies contributes but little to the further purification of the product and may be omitted. The products we obtained by salting out solutions which were oxidized under the conditions of test No. 5 compared favorably in practical staining performance with MacNeal's product.

It was evident that the product obtained in test No. 7 consisted principally of monomethyl thionin but that it also contained a large quantity of azure A. Preliminary attempts to separate the two products, or to obtain the lower homolog to the exclusion of the higher by using larger quantities of bichromate, proved unsuccessful, but a resort to higher oxidation temperatures, was found effective. In the method finally adopted the conditions employed were those of test No. 7, except that the concentrations were halved and the solutions heated at 115° to 120° C. for an hour in an autoclave. The oxidized solutions had absorption maxima at about 610 mμ and gave exceptionally well defined absorption curves indicative of a degree of homogeneity which was not obtained in any of the oxidations at the boiling point. When salted out the products gave the pure yellow-green color with concentrated sulphuric acid which is characteristic of the derivatives of thionin. We shall refer to this product hereafter as azure C. It is essentially monomethyl thionin, but contains appreciable quantities of both thionin and azure A.

Azure C was first tried out in practice as a substitute for azure A in MacNeal's tetrachrome blood stain. It performed excellently and apparently is fully equivalent to the azure A. The shade of the stain is possibly a little darker than that produced by azure A, but it has been commented on favorably by several users. It also has been successfully employed in a Giemsa substitute devised by one of us[6] employing the MacNeal tetrachrome dry stain in the place of Azure I, Azure-II-Eosin, etc.

Further tests were applied in the tissue stain for the simultaneous staining of cytological and bacterial elements appended hereto. This stain as originally developed made use of azure A, but it has been found that azure C affords a much stronger nuclear and bacterial stain, especially when the dye is employed in solution with disodium hydrogen phosphate to insure a pH of 7.7 to 8.0. This last condition seems to be an essential to consistent performance, probably due to the presence of some residual acid in the azure C salted out from acid solution, as such buffering is not essential in some of the purer samples or in those purified by recrystallization.

French, R. W., J. Lab. and Clin. Med. Awaiting publication.

Azure A has been used for some time in a new neuro staining technic at St. Elizabeth's Hospital, Washington, with excellent success in the differentiation of the glial and neuroglia cells as well as the demonstration of the Nissl bodies. (Personal communication, Dr. Walter Freeman, awaiting publication.) Azure A in this technic has been employed following formalin fixation, but has not been satisfactory following alcohol fixatives. Azure C in the same technic apparently is fully as effective as a stain. In contradistinction to azure A, however, it appears to work equally well following either formalin or alcohol as fixing agents. It also appears to afford a slightly more intense stain.

The relative stain values of the several homologs of methylene blue when employed with acetic-alcohol differentiation seem to be about as follows:

Thionin (Lauth's Violet); low stain value.
Monomethyl thionin; very strong stain value.
Asymmetrical dimethyl thionin; very strong stain value.
Symmetrical dimethyl thionin; has not been tested.
Trimethyl thionin; slight stain value.
Tetramethyl thionin; slight stain value.

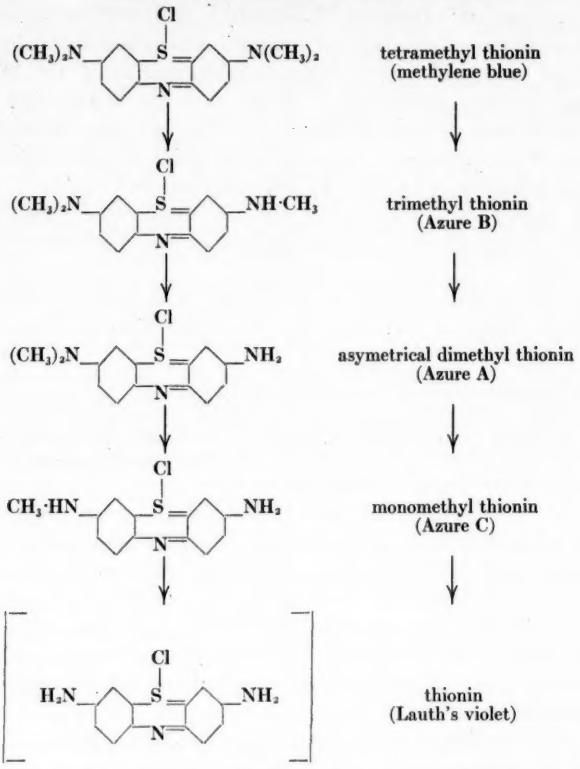
The monomethyl and asymmetrical dimethyl derivatives alone function properly in the appended stain technic, in which staining is followed by differentiation in acetic-alcohol, and the monomethyl derivative is superior. It is our expectation that the comparative testing of this and other series of homologs may eventually throw considerable light on the correlation between staining performance and chemical structure.

The acid oxidation of methylene blue is illustrated in chart 3. Although our results with acid oxidation indicate that it might be possible to effect the complete demethylation of methylene blue, we have as yet obtained only a small proportion of thionin. The possibility of this is of little practical interest, since thionin is easily obtained by synthetic methods and products of excellent quality are on the market.

Impure samples of azure B may be obtained by acid oxidation under the conditions of tests Nos. 2 and 3, or as a by-product in the preparation of azure A by adding zinc chloride to the solution from which the azure has been salted out. Our tests on the product obtained by the latter method confirmed MacNeal's preliminary verdict respecting the unimportance of the product and we have, accordingly, made no attempt to obtain the dye in a pure condition.

From the consistent results obtained in our duplicate runs under acid conditions we believe that others will find little difficulty in

CHART 3. ACID OXIDATION OF METHYLENE BLUE



duplicating the products. We have made several satisfactory runs on a semi-works scale, employing kilo lots of dye, and are convinced that the methods cited are well adapted to practical plant operation. We have, however, encountered unfavorable alterations in the character of the products occasionally in the course of their drying. We are still uncertain as to the factors responsible for these alterations, but would recommend freeing the product as completely as possible from the liquor by mechanical means before drying and hastening the drying in every way possible except by the resort to other than very moderate temperatures. We believe that the temperature in drying should not exceed 50° C.

In our opinion azure A and azure C obtained by the methods described are sufficiently pure to compare favorably with corresponding products of synthetic origin in practical application. In the

instance of the remaining intermediate product between thionin and methylene blue, the symmetrical dimethyl thionin, we intend to resort to synthesis to obtain material for comparative staining tests. So far as we are aware this compound is not formed from methylene blue by oxidation under either alkaline or acid conditions.

Azure C as a Tissue Stain

The prevailing methods of staining tissue to show both cytological and bacterial elements are either unreliable or cumbersome in practice and also require special fixing agents. The following technic has, therefore, been devised. Either azure A or azure C may be employed as nuclear and bacterial stains, azure C being preferable. For paraffin sections the detailed technic is:

1. Xylol—3 mins.
2. Absolute alcohol—3 mins.
3. 95 per cent alcohol—3 mins.
4. Water—3 mins.
5. Stain in the following for 5 minutes:

Azure C	1.5 gm.
Distilled water	100.0 c.c.

Note: As a pH of 7.5 or greater is necessary for consistent results one per cent of di-sodium hydrogen phosphate has been added to the azure. This has been found to be an essential as in commercial production it is found that there often remains in the dry dye quite a little residual acid which must be neutralized. The presence of this phosphate does not interfere with the utility of the azure in other combinations

6. Absolute methyl alcohol—5–10 seconds.

Note: This is an important step preparatory to differentiation. The sections should be left in the methyl alcohol until no more free blue is apparently washed out, which is ordinarily accomplished in a few seconds. In case the final result shows a lack of sharpness of nuclear and bacterial staining, it is probably due to improper treatment in this step.

7. Differentiate and counterstain in:

Eosin Y and orange II mixture	0.050	gm.
Glacial acetic acid	1.0	c.c.
Absolute ethyl alcohol	99.0	c.c.

Note: Carry this step until no more free blue is washed out of the section—generally 30–40 seconds. With steps 5 and 6 properly accomplished, it is difficult to over-differentiate and little fear need be entertained in that direction.

The eosin Y and orange II mixture referred to, is a mixture of eosin Y (C.I. 768) and orange II (acid orange Y, C.I. 151).

8. Absolute alcohol—1–2 mins.
9. Absolute alcohol—1–2 mins.
10. Xylol—1–2 mins.
11. Xylol—1–2 mins.
12. Xylol balsam.

In practice the writers employ Coplin jars in this staining technique, using the staining solution and alcohols for a considerable period of time. There seems to be little deterioration in the solutions, some apparent discoloration not interfering with consistent results.

This stain has been employed successfully following formalin and acetic-alcohol fixation. At first, difficulty was experienced in staining tissue which had been fixed for more than 48 hours in formalin. The addition of the sodium phosphate to the azure stain, however, removed that difficulty. The stain with this modification has also been used successfully with tissue which has been preserved in Kaiserling for some years, affording an excellent nuclear and bacterial stain simultaneously in tissue from a case of pneumonic form of Bubonic Plague.

Nuclear detail is particularly well brought out while with the recommended counterstain collagen is stained orange and the remaining cytoplasmic material a light pinkish orange. The stain affords excellent material for photography.

INHIBITORY PROPERTIES OF ANILIN DYES

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Cornell Univ. Medical School, N. Y. City[1]

ABSTRACT.—**At least four phases of bacterial inhibition have been observed in the case of bacteria exposed to the action of gentian violet and related dyes: cessation of motility; inhibition of reproduction; suspension of animation; and inhibition of sporulation. Anilin dyes may show these four types of inhibition without killing the bacteria on which they are acting. There is no doubt, however, that some of the dyes, notably the acridine group and the basic triphenyl-methanes, are capable of killing some bacteria. The difficulty of distinguishing between death and inhibition of growth has led the writer to use the term "bacteriostasis" to describe the result which dyes produce. In the case of the triphenyl-methane dyes, Gram-positive bacteria are, as a rule, much more susceptible than the Gram-negative.**

The anilin dyes have aroused interest in medicine not only because some of them are of practical use in the solution of certain laboratory and clinical problems, but also because they provide a peculiarly valuable instrument of observation for the various phases of bacterial inhibition. The study of bacteriocidal agents has long ceased to concern itself solely with the destruction of bacteria by substances which are equally destructive to tissue, and attention is now being generally focused on the investigation of substances which have predilections in this field. In the laboratory study of bacteria by cultural methods, more and more emphasis is continually being placed on fine differences in environment (that is to say, media) by means of which finer and finer distinctions can be drawn between closely related microörgaisms. In both of these fields, study of the triphenyl-methane dyes has resulted in some advance. Thus it has been shown that gentian violet is capable, when applied to certain types of surface infection, of sterilizing the surface without appreciable harm to the body cells; and by the use of gentian violet in media the possibility has been established of isolating (so delicate may the selective powers of the dye be) two strains from a "pure culture" of B. coli, one of which will grow in the presence of gentian violet, while the other will not; two strains which are in every other respect identical. By the use of the anilin dyes, therefore, we are able to study in detail some of the processes of selective inhibition

[1]Contribution from Laboratory of Experimental Therapeutics

and thus to gain closer insight into the underlying problem of bacterial growth.

The dyes also enable us to view at close range the various phases of bacterial inhibition. Even with the little that is now known in this field, by a proper choice of organism and dye, we are able more or less at will, after initiating inhibition, either to allow it to proceed to death of the organism or to check it in one of the preceding phases and thus to observe the whole process.

At least four phases of bacterial inhibition have been observed in experiments in which bacteria were exposed to the action of gentian violet and related dyes.

1. Cessation of Motility. On a cover glass vaselined by the Barber technic a droplet of actively motile *Bacillus subtilis* suspension (a 3-hour culture) is deposited from a Barber pipette and immediately adjacent to it but not touching it a similar droplet of 1–250,000 aqueous solution of gentian violet. The cover glass is placed on a depressed slide, sealed and watched on the warm stage thru an immersion lens. As moisture slowly collects on the under surface of the cover glass the two droplets gradually coalesce. When the dye touches the bacterial suspension, motility of the bacteria at the edge of the droplet immediately lessens and soon ceases. As the stain spreads into the bacterial suspension all the bacteria in the suspension droplet cease their motion and the progress of the stain can be readily followed by the behavior of the bacteria. It is interesting to note that tho the dye is distinctly hostile to the organisms they show no tendency whatever in their active motions to swim away from it but, on the other hand, swim directly into it.

2. Inhibition of Reproduction (Genesistasis). So sensitive is *B. subtilis* to gentian violet that many, if not all, the vegetative forms which have ceased their motions in the experiment just described are proved by cultural experiments to have been killed. But if acid fuchsin—a member of the triphenyl-methane series less potent for *B. subtilis* —be used, and the vegetative forms be exposed to this dye in a strength of about 1–100, fision may be prevented without killing the organism. Bacterial counts made from such a preparation will show that no growth has occurred, yet if the organisms be removed even after several days' exposure to the dye, it will be found that they are still alive. When planted on agar by the single cell technic (in order to use only vegetative forms and no spores) growth will take place.

3. Suspension of Animation. For this experiment the highly virulent *B. anthracis* and the highly sensitive mouse were used.

Thick suspensions of the organism were treated with gentian violet (5 drops of a saturated aqueous solution to 1 c.c. of bacterial suspension) and injected into the skin under the tail of a mouse. The controls all died of anthrax within the first 48 hours. The mice receiving stained organisms usually died, but death was much delayed (in one instance for 26 days) and an occasional mouse survived entirely. When the experiment was transferred to the less susceptible rabbit the animals often survived, although the controls always

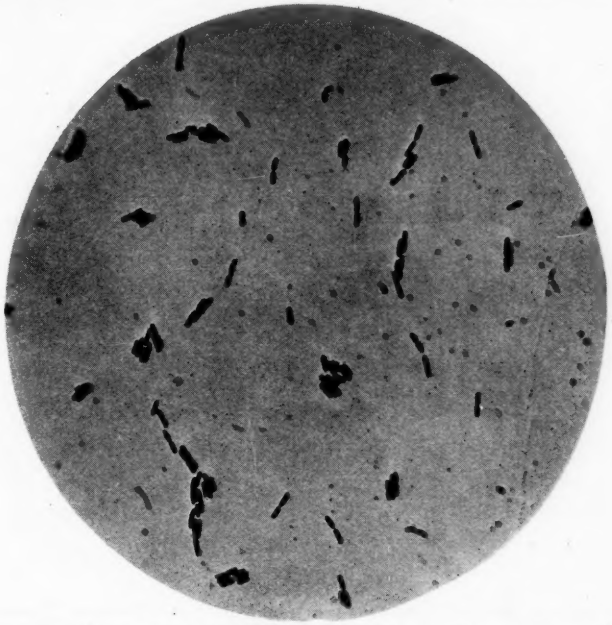

FIG. 1. Smear from suspension of B. subtilis at start of experiment. Practically all the organisms are vegetative forms.

succumbed in five to six days. The survival in perfect health of some of the mice for a period as long as 3½ weeks with ultimate death from anthrax indicates an interesting suspension of bacterial activity caused by the dye.

4. Inhibition of Sporulation. This property has recently been observed in experiments made with acid fuchsin (see Figs. 1–3). From 3-hour agar cultures of *B. subtilis* which consisted almost entirely of vegetative forms, a suspension was made in distilled water. One c.c. of this suspension was placed in each of two tubes. One tube was used as a control; to the other, 10 drops of a 1% aqueous

solution of acid fuchsin (Grübler) were added and the tubes were kept at 55°C. for one hour. They were then allowed to stand at room temperature and observations made over a period of 106 days. In the control tube spores began to form promptly. At the end usually of 24 hours to 48 hours, smears made from this tube consisted entirely of spores. In the acid fuchsin tube no spores whatever, or only an occasional one, could be observed; the smear from this tube

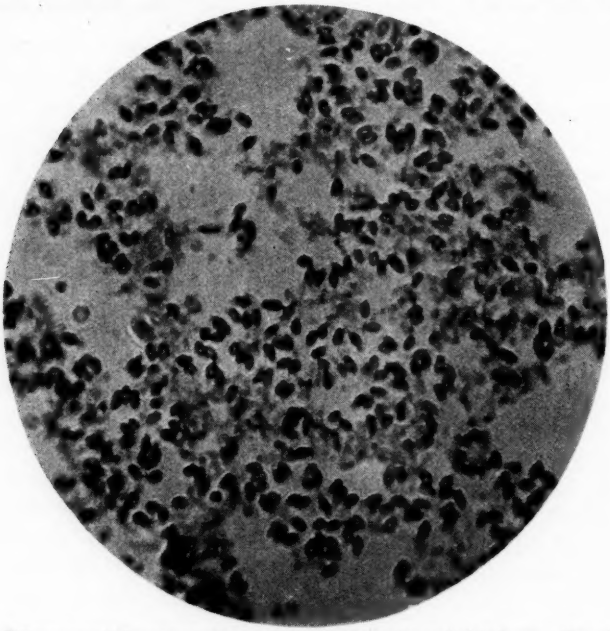

FIG. 2. Smear made from control tube of B. subtilis at the end of 48 hours. Practically all the vegetative forms have disappeared and only spores are now to be seen.

at the end of 23 days looked exactly as it did at the start of the experiment, that is to say, it consisted almost entirely of vegetative forms. That the dye had been hostile to the organism was shown by the genesistasis which it caused, for bacterial counts showed that no growth whatever had occurred. Indeed the vegetative forms not only failed to divide, but slowly died. This was proven not only by the decreasing bacterial counts, but also by making single cell transplantations (in order to insure planting only vegetative forms without spores) at various intervals of time. On the 5th day these transplantations were all positive; that is to say, the organisms were alive but had not sporulated. On the 10th day about one-half the

transplantations were positive, on the 23d day all of them were negative. The environment was therefore proven to be hostile, yet vegetative forms failed in such an environment to sporulate. This is another bit of evidence against a teleological conception of sporulation as nothing more than a protective mechanism. The process must be ultimately a chemical one. It is true that the chemical stimulus which initiates it is often hostile to the organism and the mechanism then has the appearance of protection; but a chemical

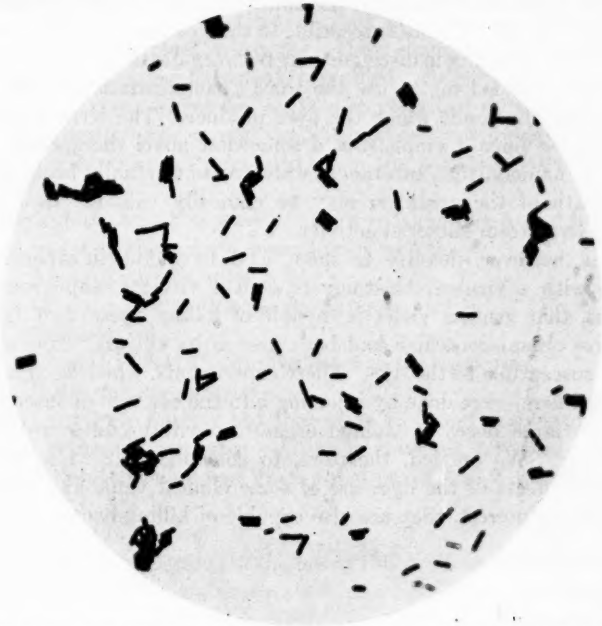

FIG. 3. Smear from tube containing B. subtilis suspension to which acid fuchsin had been added. Smear made after the organisms had been exposed to the dye for one month. Suspension was centrifugated before the specimen was taken for the smear in order to get as many bacteria in the field as possible. Notice that there has been practically no formation of spores (cf. with Fig. 1).

stimulus may be hostile to the organism (as in the experiment just cited) and at the same time inhibit the very sporulation which would protect bacteria against it.

5. It is interesting that the anilin dyes may inhibit the four phases of bacterial activity to which attention has just been called, without killing the organism. The experiment may be so conducted that one or all of these forms of inhibition may be produced at will only to have the bacteria, once they are released from the influence of the

dye, resume all their normal activities. It is not so easy as might at first appear to devise an experiment which will prove beyond a doubt that bacteria apparently killed by the dye are really dead and many of the conclusions drawn in this field have been erroneous because bacteria whose activities had been profoundly impeded were supposed to have been killed. There is no doubt, however, that some of the dyes, notably the acridine group and the basic triphenyl-methanes, are capable of killing some bacteria nor of the fact that in the case of the basic triphenyl-methanes the Gram-positive organisms are, as a rule, much more susceptible to the dye than the Gram-negatives. This difficulty in distinguishing between death and inhibition of organisms has led me to use the word "bacteriostasis" to describe accurately the result which the dyes produce. The term is also of importance since it emphasises a somewhat novel therapeutic conception, namely, that substances which do not actually bring about the death of the organism may be clinically valuable thru their power to restrain bacterial activity.

It is, however, possible to show, as I have done in experiments made with a virulent blastomyces, and a virulent staphylococcus aureus, that gentian violet is capable of killing these two Gram-positive organisms which had been proven by cultural experiments to be susceptible to the dye. These experiments, which need not be detailed here, were done by injecting into the ear vein of susceptible animals large doses of stained organisms with no untoward result whatever. We are led, therefore, to conclude that while the inhibitory effects of the dyes are of some clinical value and of great theoretical interest, they are also capable of killing bacteria.

CRYSTAL VIOLET AND ERYTHROSIN IN PLANT ANATOMY

GEMMA JACKSON
Cornell Univ., Ithaca, N. Y.[1]

Many of the microtechnical methods which have long been employed in plant anatomy depend for their effectiveness upon the unlike staining properties of lignified and non-lignified cell walls. Failure to obtain the desired differentiation of these two types of elements is very frequent when the xylem elements of the vascular bundles are either small or weakly lignified. In such cases, the combination of crystal violet and erythrosin described in this note gives excellent results. For several years it has been proving invaluable in studies on floral anatomy carried on in the botanical laboratory of Cornell University.

Although the schedule must be varied considerably to suit different lots of material, the general procedure is as follows:

1. Remove paraffin with xylol.
2. Remove xylol with absolute alcohol.
3. 95% alcohol.
4. Crystal Violet, 1% in distilled water, 15 minutes.
5. Rinse quickly in water.
6. Dehydrate quickly but thoroly in 95% and absolute alcohol.
7. Erythrosin, saturated solution in clove oil, 1 to 5 minutes.
8. Absolute alcohol and xylol, equal parts, 1 to 2 minutes.
9. Xylol.
10. Mount in balsam.

The principal variations in the use of this method are in the length of time for which the stains are allowed to act. The xylem elements in some materials stain satisfactorily in a very few minutes; for other materials a half-hour or more may be necessary. The erythrosin not only stains the non-lignified tissues but tends to replace the violet in the lignified walls; hence its action must be carefully controlled.

One advantage of this method is that it makes possible the staining of a large number of slides in a shorter period of time than is practicable with other stains. Its principal value, however, lies in its ability to give a sharp differentiation of weakly lignified and non-lignified tissues when other combinations fail. It is particularly useful for general differentiation, vascular structures being readily distinguishable from the surrounding tissues under low magnifica-

[1]Contribution from Dept. of Botany.

tions. For the finer structural details of the lignified elements, however, the violet is not so precise as certain other stains, notably safranin.

Crystal violet samples differ in their behavior in this technic and only one has so far given entirely satisfactory results. Only one erythrosin has been tried: an erythrosin B (Colour Index No. 773).

STAINING PROTOZOA WITH JANUS GREEN B.

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The use of janus green to stain mitochondria has long been known. While using it to study the mitochondria in *Trichomonas buccalis* it was found to stain the flagella also. This is an easy and quick method of determining the number of anterior flagella of the trichomonads and so of distinguishing the trichomonads from the pentatrichomonads in intestinal infections. This stain proved so useful as a flagella stain that it was applied to numerous protozoa with interesting results.

A .5 per cent aqueous solution of janus green B was diluted with normal salt or distilled water according as to whether parasitic or free living protozoa were to be studied. The dilutions used were one drop of .5 per cent aqueous solution to 10 or 20 drops of water. The stain was added either directly to the drop of culture and then covered with a cover glass, or it was placed at the side of the cover glass and drawn under by capillary attraction.

Among the flagellates studied, Trichomonas, Chilomonas and many of the Euglenoids showed blue green flagella after treatment. In Gonium the flagella took the stain and also the cytoplasmic network which holds together the organisms. The flagella of some of the Dinoflagellata also stained.

Among the Ciliata many things of interest were quickly stained. Paramecium discharged its trichocysts which stained deeply. Its macronucleus colored slowly. Usually the micronucleus could be seen unstained but very distinct. The cilia do not stain easily unless a more concentrated dye is used. However their basal granules stain and it is easy to trace the longitudinal rows of basal granules and to see the net work connecting them. In division the macronucleus and the undulating membrane in the oesophagus stained well. In conjugation many of the nuclear stages were seen clearly, i. e. the crescent stage with its division center; the micronucleus after the first maturation, with the chromosomes stained blue green on a clear spindle; and many other stages.

In both Stylonichia and Euplotes the macronucleus stains deeply and the anterior and posterior cirri color and then break up into many small cilia. The long cilia around the peristome behave in the same way. In Colpoda the macronucleus and micronucleus both stained. In Colpidium only the macronucleus stained.

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[1]Contribution from Dept. of Anatomy, School of Medicine.

Janus green B also stained the trichites and "baskets" in the oral region of several ciliata studied.

In dilutions of 1–10 a .5 per cent aqueous solution of janus green B is very toxic for most protozoa. Some are more susceptible than others. *Paramecium bursaria* is more sensitive than *Paramecium caudatum*. The stain first paralyzes the animal, which discharges trichocysts if it has them. Then it stains an increasingly deep blue green until all the structures are indistinct from the deep stain.

If however a dilution of 1–20 of a .5 per cent aqueous solution of janus green is used and the preparation is watched for some time, perhaps sealed with vaseline to prevent evaporation (though this is not necessary for the end result), the protozoa gradually become a pale blue while the food vacuoles are full of blue-stained bacteria in a pink fluid. The blue gradually changes to pink. The animals still move about and the contractile vacuoles contract normally. Eventually the animals become a deep pink and all color disappears as they die. This is a good example of the way cells reduce janus green B, first to diethylsafranin and then to a leucobase.

FUCHSIN AND PICRO-INDIGO-CARMINE, A POLYCHROMATIC STAIN FOR VERTEBRATE ORGANOGENY

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The writer has been using for some time a modification of a stain described by Ramon y Cajal (Elementos de Histologia, 1897), which differentiates the developing tissues of vertebrate embryos very successfully. The method as originally set forth is to stain sections strongly in a saturated aqueous solution of magenta red (i. e. basic fuchsin), rinse in distilled water until no more color washes out, stain in a solution of 0.25 gr. of indigo-carmine in 100 gr. saturated aqueous solution of picric acid for five to ten minutes, wash in weak acetic acid, water, absolute alcohol to remove the excess of picric acid, clear and mount.

This technique gives excellent results when used for the original purpose, i.e., of distinguishing connective tissue in histological preparations, for this stains a light blue in contrast to the green of muscle cells. After many experiments with this method on different objects, protozoa, germ cells, etc., the writer discovered that alterations in the proportions of indigo-carmine to picric acid and variations in time of staining in the different solutions altered the staining reactions profoundly. The purpose of this note is to call attention to one modification which has proved very helpful in the preparation of vertebrate embryos.

As used in the writer's laboratory, the method has been altered as follows: stain in saturated aqueous solution of basic fuchsin (syn. magenta red) 20 minutes; then, without rinsing, place in a mixture of equal parts of a saturated aqueous solution of indigo-carmine and a saturated aqueous solution of picric acid for five minutes. From the picro-indigo-carmine the slide is transferred to 70% alcohol where it is kept until the sections appear pink to the eye, a few seconds only. From this the slide is passed rapidly thru 95% alcohol to absolute in which it remains for a few seconds until the sections appear blue or green to the naked eye, when the slide is transferred to xylol. At this point the sections may be examined under the microscope and if necessary carried back to the alcohols for further differentiation of the fuchsin in absolute, or of the indigo-carmine in 70%.

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[1]Contribution No. 272 from Zoological Lab.

The stain is most effective in the case of larger embryos in which the organ systems are forming. The best results are obtained after fixing agents containing formalin. In a 23 mm. embryo of *Necturus maculosus*, fixed in Bouin's fluid, embedded in paraffin and sectioned at 20μ, the stain gave the following results in the hands of one of my students, Mr. Richard L. Webb, to whom I am indebted for the list following.

Cartilage light pink, muscle grass green, bone dark blue, precartilage light blue, connective tissue blue-gray (fibres, when present, purple), blood dark green, glandular tissue light blue, nerves dark purple to light green, cell-nuclei ordinarily dark blue but chromatin of blood corpuscles and in mitotic figures dark red. The lens of the eye was yellow.

Different shades and nuances are obtained after other fixing agents but always sharply differentiated according to the type of tissue present. If not unduly exposed to light the preparations are reasonably permanent (five to ten years).

A TECHNIC FOR PREPARING FROZEN SECTIONS

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Nothing very different or new is offered. The technic developed here for preparing permanent frozen sections varies, however, in some particulars from the usual published methods[2] and has proved successful. Most of our mounts are permanent for record, and are ready for examination the day following the removal of the tissue. Only occasionally is it necessary for a "rush" diagnosis.

Tissues, following the microscopic examination, are placed in formalin solution, 10 per cent, over night. This method of hardening has given better preparations in this laboratory than the methods of rapid hardening by heated formalin solutions. Before cutting, small pieces of the tissue are placed in distilled water. Again better preparations have been obtained with distilled water than when ordinary tap water is used. The reason for this is not known. It is thought that the poorer staining when tap water is used may be due to its reaction or to the fact that our tap water supply is chlorinated (sometimes heavily so). While small sections are usually advised and are in general easier to handle, there is no difficulty in cutting and staining sections of two or three centimeters diameter, or as large as the microtome chamber will accommodate.

Tissues are frozen with carbon dioxide gas and sections cut as thinly as the particular tissue will allow. Incidentally, there seems no need of previous embedding in gelatin or other substances. Even small bits of tissue such as uterine curettings may be cut satisfactorily if a large drop of water is first frozen on the microtome chamber and the pieces of tissue placed upon this and frozen.

Sections are transferred from the knife to distilled water and then floated onto clean slides, with care to avoid wrinkling and tearing. The excess water is drained off and the section covered with alcohol, 95 per cent. The section is then blotted carefully with smooth filter paper and covered with a very thin solution of celloidin dissolved in equal parts of alcohol and ether. The consistency of the celloidin solution to be desired is one that when placed on the finger and

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[1]From the Laboratories of the Vicksburg Sanitarium.

[2]See Mallory and Wright. Pathological Technique, 7th Ed., W. B. Saunders Co., Philadelphia, 1918, pp. 52–55.

Haden, R. L. Clinical Laboratory Methods, C. V. Mosby Co., St. Louis, 1923, pp. 275–277.

Wood, F. C., Vogel, K. M., and Famulener, L. W. Laboratory Technique, James T. Dougherty, N. Y., 1922, pp. 11–12.

allowed to evaporate, will leave a very thin film adherent. This solution for frozen sections is made by diluting the thin celloidin solution used for the celloidin embedding method, celloidin proving more satisfactory than albumin for the reason that it is not washed off if left in water a little longer than usual. The excess celloidin is drained off and the remainder allowed to set in the air for a few seconds but not allowed to dry. As soon as the celloidin has set, the slide is placed in distilled water for a minute or two to wash away the alcohol and ether contained.

The preparation is then placed in Delafield's hematoxylin solution which, thoroughly ripened, is to be preferred to the "quick method" preparations. Sections stained with it will retain their color for years and it has given better preparations. During and immediately following the war, some of the hematoxylin obtained did not give a permanent color, but we have slides stained twelve years ago that are still good, and also slides stained since 1920 that have not deteriorated. The hematoxylin solution is used undiluted. By this method the staining is rapid and the preparation must be watched closely to avoid overstaining, especially when lymphoid tissue is present. No definite staining time can be given as nearly every lot of hematoxylin solution prepared varies in strength from the one before used. Excess hematoxylin is washed out by placing the slide for two minutes in each of two jars of distilled water. The water must be kept clear and frequently changed.

The slide is next placed in eosin solution, 0.5 per cent aqueous, for two to five minutes, depending on the rapidity with which the tissue takes up the stain. Remove to jar of alcohol, 80 per cent, for two minutes. Then place in jar of alcohol, 95 per cent, for five minutes or until no further stain comes out of the section. Remove from alcohol, drain off excess, and flood section on slide with beechwood creosote for at least 10 to 15 minutes to remove all water. The creosote may be left on the slide a much longer period if desired. Unless the water is thus all removed, the celloidin becomes cloudy on standing and renders the preparation unfit for examination. Drain off excess creosote, blot carefully with smooth filter paper, and mount in Canada balsam in xylol.

BASIC FUCHSIN AS AN INDICATOR IN ENDO'S MEDIUM [1]

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ABSTRACT.—**The writer has made an investigation of various samples of basic fuchsin for use in the Endo medium for differentiating the bacteria of the colon-typhoid group. Various different concentrations of the fuchsin samples have been used in making the media. The conclusions are as follows:**

American made fuchsins differ markedly in their alcohol solubility properties. They contain materials which are very readily soluble in 95% alcohol, but which are precipitated by sodium sulphite.

This precipitation may be prevented by increasing the dilution of the fuchsin in alcohol.

In order to secure more dependable results in the use of decolorized basic fuchsin as an indicator in Endo Agar, it is advisable to test the fuchsin in different dilutions in alcohol in order to secure a completely decolorized solution. It is also advisable to carefully test those fuchsins which decolorize only in high dilutions with a known organism in Endo agar before relying on it as a satisfactory indicator for the presence of sewage organisms.

Since 1904 when Endo published his method of detecting the presence of the colon organisms and differentiating them from typhoid organisms, basic fuchsin has played an important part as an indicator in this medium. Endo's method of using basic fuchsin for this purpose is described by him as follows:[2] "After making up a 3% lactose agar medium, which has been made alkaline by adding 10 cc. of a 10% solution of sodium carbonate, then add to each liter 5 cc. of alcoholic fuchsin; this turns the medium red; now add 25 cc. of a 10% solution of sodium sulphite, which will gradually decolorize the agar so that it will be colorless when cold. The fuchsin must be filtered before using and the sodium sulphite solution must be freshly made."

The typical reaction of the colon organism is quite marked. Its colonies are a deep irridescent red, showing the fuchsin color clearly,

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[1]Editor's note.—In general it is not planned to include in this JOURNAL articles dealing with dyes used as indicators. The use of basic fuchsin in the Endo medium, however, must be an exception to this policy. To obtain a satisfactory Endo fuchsin has proved a very baffling problem, and one which has been referred so often to the Stain Commission that papers on the subject are considered within the scope of this JOURNAL.

[2]Endo, S. Uber ein Verfahren zum Nachweis der Typhusbacillus. Centbl. f. Bakt. I Abt. Orig. 35:109. 1904.

while the aerogones colonies are a rose pink and the colonies of the typhoid-para-typhoid group remain colorless. In order that this reaction may be clear cut and easily recognized, it is essential that the medium be colorless when cold and that there be as little diffusion of color as possible in the agar surrounding the growth in the inoculated plates, if one is to obtain the best results.

The directions which Endo gave for the preparation of this medium are detailed and accurate, with one exception. He does not give the method for preparing the alcoholic solution of fuchsin. He does not state whether this solution shall be a saturated or a dilute solution. Later workers have tried to remedy this omission and have recommended a saturated alcoholic solution of basic fuchsin. In Standard Methods of Water Analysis, for 1917, 1920 and 1923, the Committee on Standard Methods have gone further and directed how this saturated solution shall be prepared. They recommend as follows: "Prepare a 10% solution of basic fuchsin in 95% alcohol, allow it to stand 20 hours, decant, and filter the supernatant fluid. This is a stock solution. Add 0.5 cc. of this stock solution to 10 cc. of a 2.5% solution of sodium sulphite. This fuchsin-sulphite solution, which should be colorless, is added to every 100 cc. of the 3% lactose agar already prepared."

This method proved satisfactory in the writer's laboratory until it was found that certain brands of fuchsin did not become decolorized in the sodium sulphite solution when prepared by this method. On the contrary they formed a thick, flocculent precipitate which did not dissolve nor decolorize on standing. The fuchsin in which the reaction was first noted was an American product and designed especially as an indicator in Endo agar.

Acting upon the theory that the amount of alcohol soluble material in this fuchsin might be greater than the fuchsins previously used, it was decided to test the fuchsin, in a series of dilutions to see if a weaker solution would be decolorized by the sodium sulphite. The fuchsin was treated as follows: 1 gram of fuchsin was added to 20 cc., 40 cc., 50 cc., 80 cc., and 100 cc. of 95% alcohol respectively. These solutions were left standing over night, 20 hours, and filtered. Then 0.5 cc. of each dilution was added to 10 cc. of a 2.5% sodium sulphite solution. In the 1 to 20 and 1 to 40 alcoholic dilutions the fuchsin was not decolorized, but the precipitate became less and less marked. The 1 to 50, 1 to 80 and 1 to 100 alcoholic dilutions were decolorized in the sodium sulphite solution. Endo agar plates were made using the 1 to 50 dilution and the characteristic reaction of the colon organisms appeared upon inoculation.

The results of this test were brought to the attention of the Chairman of the Committee on Standardization of Biological Stains, and he suggested testing other fuchsins for their ability to be decolorized by sodium sulphite. Eighteen samples have been tested in this way and the technique for making the test has been simplified as far as possible.

Each sample is made up into a saturated alcoholic solution by adding 10 cc. of 95% alcohol to 1 gram of fuchsin. This is allowed to stand for 20 hours, when the supernatant fluid is decanted off and filtered. This is the stock solution. Dilutions are made by adding 1 cc. of this saturated stock solution to 1 cc., 2 cc., 3 cc., 4 cc., and 5 cc., respectively of 95% alcohol. These dilutions were denoted 1:1, 1:2, 1:3, 1:4, 1:5, etc. Each of these dilutions is then added in 0.5 cc. amounts to 10 cc. of a 2.5% sodium sulphite solution and the results carefully noted.

As a check on this method, the same fuchsin solutions were tested by adding 0.5 cc. to 2.5 cc. of a 10% sodium sulphite solution as some workers, including Endo, recommend this method for decolorizing the fuchsin since it does not dilute the agar to the same extent as the larger amount of a weaker solution. Thirteen samples were thus tested in the stock dilution of the fuchsin. Since the variations were slight, this method was not repeated with the higher dilutions of these samples or with other samples tested. The use of the 2.5% solution of sodium sulphite in 10 cc. quantities being adhered to uniformly thruout the experiments. The other method resulted more frequently in a precipitate being formed and was not thought to be quite so satisfactory.

Table 1 shows the results of the tests of different dilutions of the fuchsin samples when 0.5 cc. of each dilution was added to 10 cc. of a 2.5% solution of sodium sulphite. The tests were rechecked several times and the results recorded were uniformly the same. The anhydrous sodium sulphite used was carefully weighed and the solutions made up with all possible precautions to render the tests accurate.

Only one of the samples decolorized in the saturated stock solution; three decolorized in a dilution of 1:1 and one of these but slowly. Three of the samples were decolorized in a 1:2 dilution; while the remainder ranged from 1:3 to 1:5 before they were considered satisfactorily colorless. The precipitates formed in the lower dilutions also differed markedly. Some were thick, flocculent and heavy, resembling the precipitate made by bichromates in H_2SO_4 while others were finely granular and left a deeply colored liquid. As a rule those fuchsins in which there was the heaviest precipitate

TABLE 1. APPEARANCE OF FUCHSIN SOLUTIONS AFTER 0.5 CC. OF EACH DILUTION NAMED HAD BEEN DECOLORISED WITH 10 CC. OF A 2.5% SODIUM SULFITE SOLUTION.

Fuchsin Sample	Sat. stock Solution	Alc. dilution 1:1	Alc. dilution 1:2	Alc. dilution 1:3	Alc. dilution 1:4	Alc. dilution 1:5
CF_1	Decolorized.					
NF_2	No ppt. Not decolorized.	No ppt. Clear, decolorized.				
NF_3	No ppt. Not decolorized.	No ppt. Not decolorized.	Clear. Decolorized.			
DF_1	No ppt. Not decolorized.	Decolorized slowly.	Decolorized promptly.			
DF_2	No ppt. Not decolorized.	Decolorized. Clear.				
DF_3	No ppt. Not decolorized.	Faint pink. Clear.	Decolorized.			
CF_0	No ppt. Not decolorized.	Not decolorized. Clear.	Not decolorized. Clear.	Pink.	Pink.	Decolorized.
NF_1	Thick, flocculent ppt. Not decolorized.	Thick, granular ppt. Not decolorized.	No ppt. Not decolorized.	Clear, brown liquid	Clear straw color.	Decolorized.
NF_4	Thick, flocculent ppt. Not decolorized.	Fine ppt. Not decolorized.	Fine ppt. Red liquid.	Clear. Brown liquid.	Pink.	Decolorized.

LF_1	Thick, flocculent ppt. Not decolorized.	Brown ppt. Not decolorized.	Brown ppt. Not decolorized.	Clear, yellow liquid.	Clear, straw color.	Decolorized.
HF_1	Thick, flocculent ppt. Not decolorized.	Brown ppt. Not decolorized.	Brown ppt. Not decolorized.	Fine ppt. Not decolorized.	Clear, yellow liquid.	Decolorized.
NF_0	Thick, flocculent ppt. Not decolorized.	Fine ppt. Not decolorized.	Fine ppt. Not decolorized.	Clear. Not decolorized.	Decolorized.	
NF_8	Thick, flocculent ppt. Not decolorized.	No ppt. Clear, deep pink.	No ppt. Clear pink.	Clear, straw color.		
PF_1	Fine, thick ppt. Not decolorized.	No ppt. Deep pink.	Distinct pink.	Straw color, pink tinge.	Faint pink.	Decolorized.
EF_{102}	Fine ppt. Not decolorized.	No ppt. Brown liquid.	Clear, straw color.			
NF_5	Thick, granular ppt. Not decolorized.	Fine, scant ppt. Deep red.	Scant ppt. Deep red.	Clear, pink.	Clear, pink tinge.	
NF_7	Thick, fine granular ppt. Not decolorized.	No ppt. Deep red.	Clear, deep pink.	Decolorized.		
NF_6	Thick, flocculent ppt. Brown liquid.	Ppt. Brown liquid.	Ppt. Brownish liquid.	Scant ppt.	Scant ppt.	Decolorized.

left a clear pink or muddy brown liquid. The precipitated material itself ranged in color from a deep red to purple and brown.

It will be quite readily seen that the basic fuchsin used in making Endo agar should be properly diluted before using if it is to be decolorized in the sodium sulphite solution.

Various makes of fuchsin differ, and different lots made by the same firm vary in their solubility in alcohol. Some of these were from different lots sent out by the same firm and the results in this test show marked differences.

The fuchsins were then tested for their indicator value in Endo agar. The lowest alcoholic dilution, which was decolorized by the sodium sulphite solution, was used in each case. The writer had found that peptone is also a factor in the diffusion of color in Endo agar,[3] so care was taken to choose such brands of peptone as affected the color diffusion the least.

After repeated tests the reaction found to give the best uniform results in this work was Ph7.4 and the agar was carefully adjusted to this reaction. The organisms used to test the indicator properties of the fuchsin were: the colon organism (whose colonies show a deep, irridescent fuchsin red); the aerogenes bacillus (which has a rose pink colony) and the typhoid and para-typhoid organisms (which have white colonies). When these reactions in the respective growths were characteristic, the fuchsin was considered satisfactory as an indicator. If the color reaction was too faint in the colon colonies or too deep a red in the aerogenes colonies, the fuchsin was pronounced unsatisfactory. Table 2 gives the results of these tests.

Several samples were tested with the different brands of peptones and the results noted in the record, when the diffusion of color may be due to the peptone rather than the fuchsin. Many of the higher diluted fuchsins did not give a characteristic, irridescent red in the colon colonies, while the aerogenes colonies remained nearly colorless. In lower dilutions the fuchsin was not sufficiently decolorized so that the agar remained red, even when cold, and was not satisfactory. In some of the lower dilutions, altho the fuchsin was decolorized, it was apparently too strong and resulted in a deep red in the aerogenes colonies and a pink in the typhoid and para-typhoid colonies.

The diffusion of color after twenty-four hours was noted in plates which had not been inoculated. The fuchsins which had not been diluted enough showed marked color diffusion after twenty-four hours in these uninoculated plates.

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[3]Manuscript in preparation.

TABLE 2. BEHAVIOR OF THE VARIOUS FUCHSINS IN ENDO'S MEDIUM AS DETERMINED BY INOCULATION WITH ORGANISMS OF THE COLON-TYPHOID GROUP.

Fuchsin sample	Alcohol dilution*	Growth reaction in Endo Agar 24 hours incubation	Color diffusion in sterile plates. 24 hours incubation
CF_1	Stock	Color reaction too strong	Very deep and rapid
	1:2	" " " weak	Very faint
NF_2	1:2	Color reaction characteristic	No color
NF_3	1:2	" " "	" "
DF_1	1:2	" " "	" "
DF_2	1:2	" " "	" "
DF_3	1:2	" " "	" "
CF_0	1:5	" " "	" "
NF_1	1:2	" " "	" "
NF_4	1:2	" " "	" "
LF_1	1:4	" " "	" "
HF_1	1:4	" " "	" "
NF_0	1:4	" " "	Usual reaction for peptone used
NF_8	1:2	" " "	" " " " "
PF_1	1:5	" " not characteristic	No color
EF_{102}	1:4	" " characteristic	" "
NF_5	1:2	" " not satisfactory	" "
NF_7	1:3	" " " "	" "
NF_6	1:5	" " " "	" "

*This represents the proportion in which the stock fuchsin solution was diluted in alcohol before decolorising with sodium sulfite.

To summarize the results recorded in Tables 1 and 2, the fuchsins have been grouped under three heads: *Good*, *Fair*, and *Unsatisfactory*. Those listed as *Good* are the fuchsins which when properly diluted were readily decolorized in the sodium sulphite solution and were uniformly stable in the characteristic color reaction of both the colon and aerogenes colonies. In the judgment of the writer, they were fuchsins best suited for routine class room work and for experimental purposes. Those listed as *Fair* are the fuchsins which were decolorized in the sulphite solution but the color reaction was not so markedly characteristic nor stable in the colonies of the aforenamed organisms. They would not be particularly desirable for class room work where the best results are needed. Those classed as *Unsatisfactory* are those which, when diluted until they were decolorized in the sodium sulphite solution, were apparently too weak to give the characteristic color in the colonies, or showed other undesirable or unstable qualities.

Good	Fair	Unsatisfactory
DF_1	NF_2	HF_1
DF_2	NF_3	LF_1
DF_3	PF_1	CF_1
NF_1	EF_{102}	NF_6
NF_4	NF_8	
CF_0	NF_7	
NF_0	NF_5	

It will be seen that of these eighteen samples tested, only 4 or 22% could not be used as indicators in Endo agar. Of these, three showed a marked precipitate in alcoholic dilutions as high as 1:2 or 1:3 when added to the sodium sulphite solution, while the fourth, CF_1, decolorized readily in the stock solution, but when used in Endo agar was very unstable. When it was used in the stock solution or 1:1 dilution it caused a deep color diffusion in uninoculated plates while a 1:2 dilution was so weak that it did not show the characteristic color in inoculated plates. Its instability was so marked that dilution between these ranges was not attempted.

Of the remaining fuchsins, 50% are *Good*, and 50% seemed *Fair*. The classification is based to some extent on the judgment of the experimenter; those classed as *Good* were so eminently satisfactory in every way that no doubt remained as to their value when the plates were studied.